

Exclusion of Polymorphisms in Carnosinase Genes (*CNDP1* and *CNDP2*) as a Cause of Diabetic Nephropathy in Type 1 Diabetes

Results of Large Case-Control and Follow-Up Studies

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OBJECTIVES—Recently, an association was found between diabetic nephropathy and the D18S880 microsatellite, located in the carnosinase gene (*CNDP1*) on chromosome 18q. Alleles of this microsatellite encode for a variable number of leucine residues (from four to seven) in the leader peptide of the carnosinase precursor. The frequency of subjects homozygous for the five leucines was higher in control subjects than in case subjects in studies focusing on type 2 diabetic patients. To test whether this finding can be extended to type 1 diabetic patients, we carried out a comprehensive study on association between diabetic nephropathy and the D18S880 microsatellite and 21 additional SNPs that tagged the genomic region containing *CNDP1* and *CNDP2*.

RESEARCH DESIGN AND METHODS—Overall, 1,269 Caucasian patients with type 1 diabetes were included in the study, including 613 patients with normoalbuminuria and a long duration of diabetes, 445 patients with persistent proteinuria, and 211 patients with end-stage renal disease (ESRD). All patients were genotyped for selected polymorphisms, the associations with diabetic nephropathy were tested by a χ^2 test, and odds ratios were calculated.

RESULTS—We did not find any significant association between diabetic nephropathy and any examined genetic markers. The negative findings of the case-control study were supported further by negative findings obtained from the 6-year follow-up study of 445 patients with persistent proteinuria, during which 135 patients developed ESRD.

CONCLUSIONS—Our large, comprehensive study did not find an association between the D18S880 microsatellite or any other polymorphisms in the *CNDP2*–*CNDP1* genomic region and susceptibility for diabetic nephropathy in type 1 diabetes. *Diabetes* 57:2547–2551, 2008

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Diabetic nephropathy is the most severe complication resulting from type 1 diabetes. Approximately 30 to 40% of type 1 diabetic patients develop this complication, which leads to end-stage renal disease (ESRD) and increases the risk of cardiovascular morbidity and mortality (1–3). Epidemiologic and family studies have demonstrated that in addition to levels of glycemic control, genetic factors play an important role in the development of diabetic nephropathy (4–6). Many groups have been searching for genes that make individuals susceptible to diabetic nephropathy using linkage studies or candidate gene approaches (7).

Recently, a family study conducted in 19 Turkish extended families exhibiting type 2 diabetes showed evidence for linkage between microalbuminuria and proteinuria and the genomic region on chromosome 18q22.3–q23 (8). Following these findings, Janssen et al. (9) found an association between diabetic nephropathy and the common sequence variant in the carnosinase 1 gene (*CNDP1*) located in that region. The case-control study, however, consisted of only 135 case and 107 control subjects (both groups consisted of type 1 and type 2 diabetic individuals) selected from among patients in four different countries. In that study, association was found between microsatellite D18S880, a trinucleotide repeat in exon 2 of *CNDP1*, and diabetic nephropathy. Because this polymorphism lies in the 5' coding part of the transcript, the number of trinucleotide repeats is directly related to the number of leucine residues in the leader peptide of the carnosinase precursor: five, six, or seven leucines. The frequency of subjects homozygous for the five leucines was higher in control subjects compared with case subjects. The odds ratio (OR) of diabetic nephropathy for carriers of four, six, or seven leucines, in comparison with those homozygous for the five leucines, was 2.6 (95% CI 1.4–4.8). Interestingly, functional studies carried out by the same group provided some support for a biological explanation of the observed association (9–11).

Recently, Freedman et al. (12) published data from a case-control study in which the authors selected case (patients with type 2 diabetes and ESRD) and control (individuals with type 2 diabetes but without diabetic nephropathy) subjects from among Caucasians in North Carolina. The cases were ascertained through dialysis centers, and the control subjects were recruited as volunteers for the Diabetes Heart Study. This study supported Janssen et al.'s (5) findings that subjects with diabetes but without diabetic nephropathy were more frequently ho-

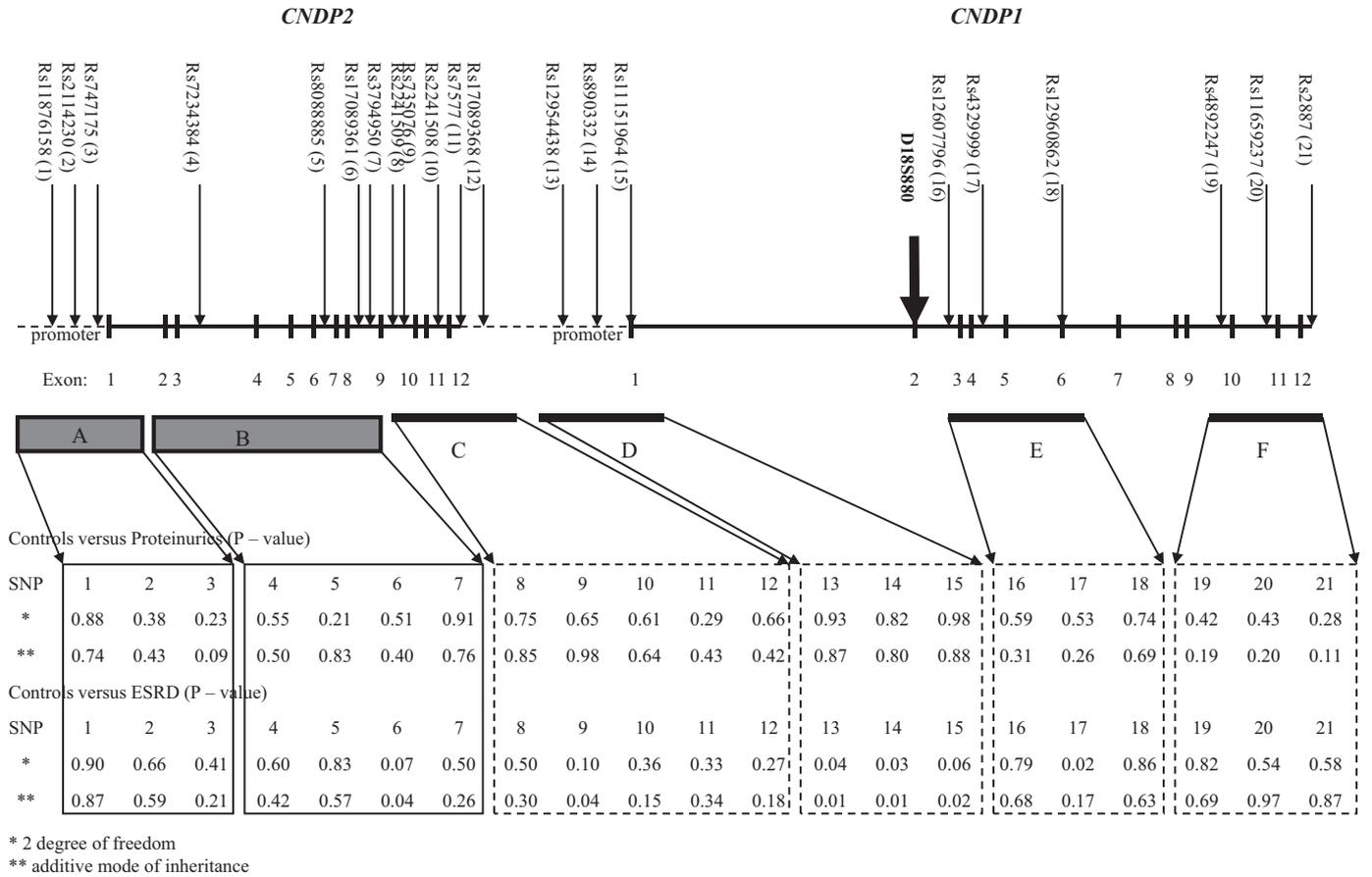


FIG. 1. Approximate genomic location and P values for selected single SNP comparisons for the CNDP1 and CNDP2 genomic regions assuming additive mode of inheritance. Nominal P values for χ^2 tests with 2 d.f. are also provided. Haploblocks are shown schematically as gray rectangles. SNPs 1–12 are considered tagging SNPs for particular haplo- and interblocks. *2 d.f.; **additive mode of inheritance.

mozygous for the five-leucine repeat genotype than those who had ESRD. Although both studies concluded that the five-leucine repeat was protecting against diabetic nephropathy, the effect of duration of diabetes on frequency of this genotype in control and case subjects was not examined in either study (13).

In this report, we attempted to extend the findings of Janssen et al. and Freedman et al. (9,12) to patients with type 1 diabetes. We examined the association between the number of leucine repeats in CNDP1 and other tagging single nucleotide polymorphisms (SNPs) in the CNDP2-CNDP1 genomic region and the risk of various stages of diabetic nephropathy in a population of Caucasian patients with type 1 diabetes living in the eastern part of Massachusetts and attending a large diabetes center. Large case-control as well as follow-up study designs were used.

RESEARCH DESIGN AND METHODS

Patients for this investigation were selected from the Joslin Study on Genetics of Diabetic Nephropathy in type 1 Diabetes. The detailed description of the Joslin clinic population and the method of selection of patients for the study can be found in the online appendix (available at <http://dx.doi.org/10.2337/db07-1303>). The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol and informed consent procedures for the study.

Diagnosis of type 1 diabetes and diabetic nephropathy. Patients were considered type 1 diabetic if hyperglycemia was diagnosed before age 40 years, its control required insulin treatment within 1 year of diagnosis, and such treatment could not be interrupted thereafter. Only patients aged 18–59 years at examination were eligible for enrollment into these studies. The diabetic nephropathy status of each patient was determined on the basis of medical records and measurements of the urinary albumin-to-creatinine ratio. Methods for measuring the albumin-to-creatinine ratio and classification

criteria have previously been described (14). At the time of enrollment, patients were classified into the following study groups (detailed description of the criteria can be found in the online appendix): 1) Control subjects were considered to have normoalbuminuria if they had diabetes duration of ≥ 15 years and had persistent normoalbuminuria; 2) Case subjects were considered to have persistent microalbuminuria if they had diabetes duration ≥ 3 years and had persistent microalbuminuria; 3) Case subjects were considered to have persistent proteinuria if they had diabetes ≥ 7 years and had persistent proteinuria; and 4) Case subjects were considered to have ESRD if they had begun dialysis or received a renal transplant as a result of diabetic nephropathy. Patients with proteinuria at the enrollment were followed until 2005 to determine who progressed to ESRD.

DNA was extracted using a standard phenol-chloroform protocol. For the current study, we used only patients classified as control subjects ($n = 613$) or case subjects with advanced diabetic nephropathy (445 with proteinuria and 211 with ESRD). Patients with microalbuminuria were not examined in the current investigation. In total, 1,269 patients from the Joslin Study on Genetics of Diabetic Nephropathy in type 1 Diabetes were used in this investigation.

D18S880 microsatellite was genotyped using previously described primers (9) and standard PCR protocols. The PCR products were separated using the ABI 377 Sequencer (Perkin Elmer, Foster City, CA). All samples were successfully genotyped for microsatellite markers. All selected SNPs were genotyped using Sequenom MassArray Platform (Broad Institute Center for Genotyping and Analysis, Cambridge, MA). The maximum SNP-specific missing data rate was 4.7%.

Selection of SNPs. We identified haploblocks in the CNDP2 gene locus with the D' 90% confidence bounds method of Gabriel et al. (15) using Haploview software (16). We selected tagging SNPs (htSNPs) needed to tag frequent haplotypes in a block (frequency ≥ 0.05) using the method of Stram et al. (17). The minimum set of htSNPs within each block was selected to ensure an R_h^2 of at least 0.80 for all haplotypes observed at a frequency of at least 5%. In this set of htSNPs, the minimum R_h^2 was always ≥ 0.80 . For the CNDP2 locus, we selected 12 htSNPs, which cover two haploblocks denoted as A and B and one inter-block denoted as C. Because of less clear haploblock structure in the

TABLE 1

Selected clinical characteristics of patients in the study group according to nephropathy status at time of enrollment into the study

	Normoalbuminuria	Proteinuria	ESRD
<i>n</i>	613	445	211
Men (%)	46.8	53.5	56.9
Age at diabetes diagnosis (years)	13.2 ± 7.4	12.0 ± 8.0	11.6 ± 6.3
Duration of diabetes (years)	21.8 ± 9.7	24.9 ± 8.5	31.0 ± 7.3
Age at examination (years)	35.0 ± 9.2	38.4 ± 8.8	42.6 ± 6.6
A1C (%)	7.8 ± 1.2	9.0 ± 1.7	8.1 ± 2.0
Systolic blood pressure (mmHg)	118 ± 13	134 ± 18	134 ± 20
Diastolic blood pressure (mmHg)	71 ± 8	79 ± 10	76 ± 11
BMI (kg/m ²)	25.0 ± 3.5	26.1 ± 5.4	24.5 ± 4.9
Median ACR (mg/g)	10.7 ± 5.2	1,402 ± 1,337	—

Data are means ± SD unless otherwise indicated. ACR, albumin-to-creatinine ratio.

CNDP1 locus, we selected all available SNPs from the PubMed database with minor allele frequency >5%. This included two SNPs used previously by Janssen et al. (9). In total, we genotyped one microsatellite located in exon 2 and 9 SNPs in the *CNDP1* locus. These SNPs were grouped into three loosely defined haploblocks (Fig. 1).

Power calculations. To calculate the sample size required to replicate the association of leucine repeats with diabetic nephropathy, we assumed a protective allele frequency of 59% and a genotype relative risk (RR) of 2.56 (estimated based on OR) for individuals who are not five-leucine homozygotes (9). We assumed a diabetic nephropathy prevalence of 30%. Under these assumptions, we estimated that 613 control and 656 case subjects would provide >99% power to confirm the findings reported by Janssen et al. (9). On the other hand, if we used 211 ESRD case and 613 control subjects we would have had 80% power to detect an OR of 1.61, assuming the recessive model. OR 1.51 for the dominant model, or OR 1.28 for the additive model at the $P = 0.05$ level of statistical significance. Power calculations were performed with the program CaTS of Skol et al. (18) (available at <http://www.sph.umich.edu/csg/abecasis/CaTS/>). In our follow-up study, we were able to detect a genotype RR of 1.5 with a power of at least 80% at the $P = 0.05$ level of statistical significance. To calculate power in the follow-up branch of our study, we used the PS program of Dupont and Plummer (19) (available at <http://www.mc.vanderbilt.edu/prevmed/ps/index.htm>).

Statistical analysis. The data from the study were analyzed using SAS (version 8.02; SAS Institute, Cary, NC). Deviations from Hardy-Weinberg equilibrium were evaluated with a χ^2 goodness-of-fit test. For single-marker analysis, we used SnpGwa, version 1.0, software under assumptions of additive models. We tested the association between the genotype polymorphisms and diabetic nephropathy using a χ^2 test and calculated ORs. The Haplo.stat package was used to infer and compare haplotype distribution with the skip.haplo option set to 0.05 (20).

In the follow-up study, we calculated life tables to compute incidence rates. The observation ended at the year of the development of ESRD, death, loss to follow-up, or 2005. Additionally, a Cox proportional hazards model controlling for several covariates was constructed to examine the effect of genotypes on risk of ESRD.

RESULTS

Characteristics of the study groups are shown in Table 1. They reflect the study design. Age at diagnosis of type 1 diabetes was similar in all groups, all patients had long duration of diabetes, all were treated with insulin (data not shown), and all had similar A1C and BMI. However, patients with proteinuria and ESRD had longer duration of diabetes (and therefore were older) and higher blood pressure than control subjects.

Figure 1 shows the genomic region that contains *CNDP2* and *CNDP1*. Both genes are located on the long arm of chromosome 18 (18q22.3) in a tail-to-head orientation. *CNDP2* spans 24.8 kb and has 12 exons. *CNDP1* is 13 kb telomeric from *CNDP2*, which spans 50.6 kb and also has 12 exons. The linkage disequilibrium (LD) structure of *CNDP2* allows us to distinguish two haploblocks (A and B) followed by an interval (C) without haploblock structure. *CNDP1* is characterized by a less clear haploblock struc-

ture, and the selected SNPs were grouped into three intervals: D, E, and F. Overall, in the *CNDP2-CNDP1* genomic region, we selected and genotyped 21 SNPs and the D18S880 microsatellite. All SNPs were in Harvey-Weinberg equilibrium.

The results of single SNP analysis are shown in the bottom part of Fig. 1 and in an online appendix. None of the examined SNPs appeared to be significantly differently distributed between control and case subjects with proteinuria or ESRD except for SNPs located in the interval D, which contains a proximal and distal promoter of *CNDP1*. Genotype frequencies of these three SNPs were different at $P < 0.05$ between control and case subjects with ESRD. However, the differences became insignificant after Bonferroni adjustment. Haplotype analysis of these three SNPs did not yield a more significant difference between control and case subjects with ESRD (data not shown).

The results for the D18S880 microsatellite were analyzed separately. Five alleles corresponding to repeats of four, five, six, seven, and eight leucines were found. The first and last alleles were very rare (<1%). The frequency of the allele encoding the five leucines, the protective allele from the Janssen et al. report, was very similar in all three study groups: 57.8% in patients with normoalbuminuria and long-duration type 1 diabetes (control subjects) and 54.8 and 59.2% for patients with proteinuria and ESRD, respectively. The frequency of other alleles was also very similar. Overall, the distribution of the alleles did not differ among the three study groups ($P = 0.2$) (Table 2A in the online appendix).

TABLE 2

Distribution of genotypes for D18S880 microsatellite according to study groups

D18S880 genotypes	Control subjects with normoalbuminuria	Case subjects with proteinuria	Case subjects with ESRD
<i>n</i>	613	445	211
5_5	33.0	29.4	32.2
5_6	43.6	45.2	47.9
5_7	6.0	5.4	6.2
6_6	12.6	15.5	11.8
X_X	4.9	4.5	1.9

Genotype frequency data are percent. Genotypes with frequencies <5% were combined to X_X. Control subjects with diabetes duration <25 and ≥25 years had similar distribution of the D18S880 genotype. Total $\chi^2 = 7.50$; 8 d.f.; $P = 0.48$.

TABLE 3
Incidence of ESRD during the follow-up according to genotypes of the selected four markers

Polymorphism	Genotype	<i>n</i>	ESRD	PYs	IR/100 PYs	HR (95% CI)	<i>P</i>
rs12954438*	A/A A/G	101	30	516	5.8	1.0 (Ref.)	0.94
	G/G	335	103	1,885	5.5	1.02 (0.67–1.53)	
rs890332*	G/G A/G	101	30	515	5.8	1.0 (Ref.)	0.94
	A/A	334	103	1,871	5.5	1.02 (0.67–1.53)	
rs11151964*	A/A A/G	109	32	590	5.4	1.0 (Ref.)	0.86
	G/G	331	102	1,840	5.5	1.04 (0.69–1.56)	
D18S880	55	131	34	746	4.6	1.0 (Ref.)	0.15
	5X	226	82	1,196	6.9	1.34 (0.90–2.02)	
	XX	88	19	512	3.7	0.79 (0.44–1.39)	

For the polymorphism D18S880, alleles 4, 6, and 7 were combined to X. HRs presented are for each of the selected polymorphisms according to genotypes. *n* = patients with proteinuria at the entry of the study. *Due to small number of risk homozygotes, the data for the carriers of the risk allele have been combined. ESRD, new cases of ESRD; IR/100 PYs, incidence rate per 100 patient-years; PY, patient-years.

The frequencies of the D18S880 genotypes in the study groups are presented in Table 2. In this table, the genotypes less frequent than 5% were combined into one group referred to as the X_X genotype. The frequency of subjects homozygous for five leucines was very similar in the three study groups. The frequencies of other genotypes were also similar (*P* = 0.5). Combined analysis of the D18S880, in which control subjects were compared with case subjects (patients with proteinuria or ESRD), also failed to replicate results published by Janssen et al. The OR in our study was 1.13 (95% CI 0.89–1.43).

To further study the associations between ESRD and the three SNPs located in interval D with the D18S880 genotypes, we conducted a follow-up study. In this study, 445 patients with proteinuria were followed for an average of 5.6 years (range 3–16). During the follow-up, 135 patients with proteinuria developed ESRD. Table 3 shows the results of the follow-up study. Incidence rates of ESRD during follow-up were not different according to genotypes of the three examined SNPs or the D18S880 microsatellite. The Cox proportional hazards model was also constructed to examine the effect of genotypes on risk of ESRD, controlling for covariates such as sex, age at diagnosis of diabetes, duration of diabetes, and treatment with ACE inhibitors or lack thereof. This analysis also did not reveal any difference in risk of developing ESRD according to the examined genotypes (Table 3).

DISCUSSION

Recently, an association was found between diabetic nephropathy and the D18S880 microsatellite in the carnosinase gene (*CNDP1*) (9). Alleles of this microsatellite encode for a variable number of leucine residues (from four to seven) in the leader peptide of the carnosinase

precursor. Homozygotes for the five leucines were found less frequently among patients with diabetic nephropathy than among patients without it (9). To test whether this finding, which has significant biological implications, can be extended to patients with type 1 diabetes, we carried out a large comprehensive examination of association between various stages of diabetic nephropathy and the D18S880 microsatellite, as well as 21 SNPs that tagged the genomic region containing *CNDP2* and *CNDP1*. Unfortunately, we did not find any significant association between diabetic nephropathy and any of the examined genetic markers. Noteworthy, three SNPs in the promoter region of *CNDP1* (rs12954438, rs890332, and rs11151964) could be considered associated with ESRD, assuming the additive inheritance model at the nominal *P* = 0.05 significance level. However, those SNPs have never been studied before. Moreover, based on our data, those SNPs are not in LD with any of the D18S880 alleles. Therefore, we considered our examination of the SNPs in the promoter region of the *CNDP1* gene as exploratory study and employed stringent Bonferroni correction. The negative findings of the case-control study were supported further by negative findings obtained in the 6-year follow-up study of 445 patients with persistent proteinuria, during which 135 patients developed ESRD. In conclusion, our large comprehensive study did not support the role of D18S880 microsatellite or any other polymorphisms of *CNDP2* and *CNDP1* in the development of diabetic nephropathy in type 1 diabetes. Following, we discuss our results in comparison with findings from the other studies.

Table 4 shows the frequency of *CNDP1* five leucine/five leucine genotype in control and case subjects in each of the three studies. Due to the small number of individuals with type 1 diabetes in the study by Janssen et al. (9), we

TABLE 4

Comparison of frequency of *CNDP1* five leucine/five leucine repeat genotype in case (patients with diabetic nephropathy) and control (individuals with diabetes) subjects in the three studies

Study	<i>n</i>	Percent of 5–5 in control subjects	Percent of 5–5 in case subjects	OR* (5–5 as reference)
Janssen et al. (9): type 1 and type 2 diabetes**	242	43	27	2.0 (1.17–3.42)
Freedman et al. (12): type 2 diabetes	600	39	29	1.5 (1.07–2.13)
Wanic et al. (current study): type 1 diabetes	1,269	33	30	1.1 (0.89–1.43)

*95% CIs are provided in parenthesis, and ORs calculated for the protective effect. **Including 57 patients with type 1 and 185 with type 2 diabetes. 5–5, five leucine/five leucine repeat genotype.

evaluated the combined frequencies among both type 1 diabetic and type 2 diabetic patients. Interestingly, the frequency of the five/five homozygotes in case subjects is very similar in the three studies (differences were not statistically significant: $\chi^2 = 0.5$; $P = 0.8$). The frequency of the five/five homozygotes seems to be different among the control subjects, being most frequent in the Janssen et al. study, intermediate in the Freedman et al. study, and lowest in the current study ($\chi^2 = 5.6$; $P = 0.06$). The first study was the smallest and used patients from four different countries, including one Arabic country. The study by Freedman et al. (12) selected case and control subjects from among European-Americans residing in North Carolina but did so through completely different mechanisms. The case subjects were found through dialysis centers. The control subjects were recruited as volunteers for the Diabetes Heart Study. Only the current study drew case and control subjects from the same clinic population, and it included more case and control subjects than the two other studies combined.

At present, there is no clear explanation for the discrepant findings summarized in Table 4. One possibility is that they result from improper selection of control subjects in some of the studies. However, because the differences in the previous studies were observed in patients with type 2 diabetes, one cannot exclude the possibility that the five leucine/five leucine genotype of the D18S880 microsatellite plays a role in protection against diabetic nephropathy in patients with type 2 diabetes but not type 1 diabetes. Therefore, the discrepant findings may simply indicate differences in genetic susceptibility to diabetic nephropathy in type 1 and type 2 diabetes (21).

Additionally, we examined the possibility that the D18S880 microsatellite alleles are not causally related to diabetic nephropathy but are in LD with other DNA sequence differences that are causal. To test this possibility, we examined 21 SNPs that encompass most of the genetic variation in the *CNDP2-CNDP1* genomic region. Neither single-SNP nor haplotype analysis showed any significant association with proteinuria or ESRD. However, these negative findings should be qualified because the haplotype structure, particularly for *CNDP1* locus, is poorly defined in the current HapMap data. Furthermore, we had only limited information to estimate LD between the D18S880 microsatellite and other SNPs in the *CNDP2-CNDP1* genomic region.

Some other qualifications of our findings should be discussed. First, although our study is large, it is too small to detect significant ORs below 1.2. To demonstrate such a small effect, one would need several thousand case and control subjects, as was demonstrated recently in studies on type 2 diabetes (22,23). The cross-sectional part of our study might have been underpowered to detect ORs reported by Freedman et al. (12). However, this deficiency was overcome by implementing the follow-up study, which had sufficient power to detect a genotype RR of 1.5. Second, it is well known that patients with ESRD have very high mortality, and some of the associations may not be related to risk of the development of ESRD but may instead determine survival of such patients. In our study, we excluded such a possibility by examining the risk of development of ESRD according to specific genotypes in the prospective study. Finally, spurious associations or lack thereof can result from poor quality of genotyping. In our study, we used manual genotyping, which can be susceptible to errors, for D18S880 microsatellite. How-

ever, we had only a few errors when we repeated genotyping in 10% of the study population. On the other hand, tagging SNPs were genotyped on the Sequenom platform with almost perfect genotyping.

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